Mitochondrial activation in the growth-restricted fetus of monochorionic twins

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Objective: To study the regulatory mechanisms of selective intrauterine growth restriction (sIUGR) independent of confounding genetic factors, monochorionic (MC) twins are the ideal model, because they have identical genomic DNA. We hypothesize that the intrauterine growth restriction fetus has mitochondrial activation compared with its larger counterpart, and sought to demonstrate this using the MC twin model.
Design: Fetal cord blood and amniotic fluid of MC twins were prospectively collected during delivery. Mitochondrial DNA of cord blood was measured using real-time quantitative polymerase chain reaction (PCR), and mitochondria in amniotic fluid mesenchymal stem cells (AFMSCs) were analyzed with MitoTracker staining.
Setting: Tertiary referring center.
Patient(s): Forty-three pairs of MC twins, including 24 pairs with sIUGR and 19 pairs without.
Intervention(s): None.
Main Outcome Measure(s): Mitochondrial DNA contents were measured and presented as fold difference between the small and large fetuses. After staining with MitoTracker, mitochondrial intensity in AFMSCs was analyzed with the Image J program.
Result(s): The fold differences of the cord blood mitochondrial DNA content between the small and large twins were significantly higher in the MC twins with sIGUR (2.5 ± 1.2, $n = 24$ pairs) than in those without sIUGR (1.2 ± 0.3, $n = 19$ pairs). In addition, mitochondrial staining intensities were significantly higher in the AFMSCs derived from growth-restricted fetuses than from control fetuses.
Conclusion(s): Mitochondrial activation in the sIUGR fetus of MC twins was likely regulated by locally adverse placental and blood flow conditions, instead of genetic factors. (Fertil Steril® 2013;100:241–6. ©2013 by American Society for Reproductive Medicine.)
Key Words: Mitochondrial DNA, monochorionic twins, intrauterine growth restriction (IUGR)

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Mitochondria are specialized energy-converting organelles. Without mitochondria, cells depend on low-efficiency, anaerobic glycolysis to produce adenosine triphosphate (ATP). Every mitochondrion contains 2–10 copies of mitochondrial DNA (mtDNA) in its matrix, resulting in more than 10,000 copies of mtDNA in each human cell (1–3). Mitochondrial DNA typically replicates during every cell cycle, and each daughter cell maintains a constant amount of mtDNA (4). However, replication of mtDNA may also be regulated by genetic (5) and environmental factors, such as oxidative stress (6, 7). In addition, changes in mtDNA content are associated with numerous diseases, including type 2 diabetes mellitus (8), cardiovascular disease (9), and polycystic ovary syndrome (PCOS) (10). Relevant to maternal-fetal pathophysiology, mtDNA content is increased in the placental cells of growth-restricted pregnancies (11) and in the peripheral blood of mothers with intrauterine growth restriction (IUGR) babies (12). However, the regulatory mechanisms of mitochondrial and mtDNA replication in growth-restricted fetuses are largely unknown.

Monochorionic (MC) twins are a subtype of monozygotic twins where both fetuses have identical genomic DNA and grow in the same maternal

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environment. Therefore, the presence of one IUGR twin in MC twins is an ideal model to investigate the mechanisms of IUGR pathophysiology, independent of confounding genetic factors. We hypothesized that fetuses with IUGR would demonstrate enhanced mitochondrial activation compared to fetuses without IUGR. We tested this hypothesis by measuring mtDNA content in MC twins with and without discordant twin growth.

Two unique observations have been reported in the babies with IUGR: the early occurrence of catch-up growth during the postnatal period (13, 14) and the late development of metabolic diseases during adulthood (9, 15–18). Our findings of activated mitochondria in the selective IUGR (sIUGR) fetus of MC twins may provide a mechanistic link between these two phenomena.

MATERIALS AND METHODS

Patients

We enrolled mothers with known MC twin pregnancy who delivered at Chang Gung Memorial Hospital, Linkou Medical Center, Taiwan. Exclusion criteria of this study included obstetric complications and maternal systemic diseases, such as hypertension, preeclampsia, gestational diabetes, or twin–twin transfusion syndrome. An informed consent was obtained from each pregnant woman before she participated in the study. This study was approved by the Chung Gung Memorial Hospital Institute Review Board (96-1279B and 98-0669B).

In this study, IUGR was defined as an estimated fetal weight below the 10th percentile on a standardized birth weight chart for a singleton pregnancy (19). Fetal weight discordance (%) between twins was calculated by 100% × [(Birth weight of the larger twin) - (Birth weight of smaller twin)] / Birth weight of the larger twin. Two criteria were used to identify a sIUGR in MC twins: fetal weight discordance >25% and the presence of IUGR in the smaller MC twin (20). The chorionicity of the twins was determined by an experienced sonographic specialist in the first or early second trimester and confirmed by a placental examination after delivery.

Collection of Blood Specimens

Individual umbilical blood specimen was collected from the umbilical vein of each twin immediately after delivery. All blood specimens were collected into tubes containing 1.0 mg/mL disodium ethylenediaminetetraacetic acid (EDTA). Total DNA was extracted from cord blood using QIAamp DNA Mini Kit Q (Qiagen) and stored at −80°C.

Isolation and Culture of Amniotic Fluid Mesenchymal Stem Cells

Amniotic fluid was collected from each of MC twins during delivery. The procedures of amniotic fluid mesenchymal stem cell (AFMSC) isolation and culture were previously reported (21). Briefly, AFMSC were cultured in α-minimal essential medium (MEM), which was supplemented with fetal bovine serum (Hyclone) and basic fibroblast growth factor (R&D Systems), and incubated in filtered air with 5% CO_2 at 37°C. After nonadherent cells were removed, the culture medium was changed every 3–4 days.

Characterization of AFMSC

The human AFMSCs were analyzed for cell surface markers with flow cytometry using fluorescein isothiocyanate (FITC)-conjugated or phythoerythrin-conjugated antibodies (BD Biosciences/Southern Biotech), as previously reported (21, 22). For flow cytometry analysis, cells were detached with treatment of trypsin/EDTA in phosphate-buffered saline (PBS), washed with PBS, and incubated for 30 minutes with the designated antibodies. For each sample, 1×10^4 flow cytometry events were obtained and analyzed using Cell Quest software (Becton-Dickinson).

Molecular Confirmation of the Monozygosity in Each Pair of Monochorionic Twins

A total of 15 short tandem repeat markers were used to analyze the genomic DNA that was extracted from cord blood in 10 randomly selected pairs of MC twins. The markers were D8S1179, D21S11, D7S820, CSF1PO (in 5q33.3-34), D3S1358, TH01 (in 11p15.5), D13S317, D16S539, D2S1338, D19S433, vWA (in 12p12-pter), TPOX (in 2p2.3-2pter), D18S51, D5S818, and FGA (in 4q28).

Real-time Quantitative Polymerase Chain Reaction Analysis

The procedures for mtDNA quantification with real-time quantitative polymerase chain reaction (PCR) using a TaqMan PCR Core Reagent Kit and ABI 7900 Thermocycler (Applied Biosystems) were previously reported (5). The mitochondrial gene 7S encoding D-loop was used as the target gene (Hs02596861_s1; Applied Biosystems), and the amount of the glyceraldehyde-3-phosphate dehydrogenese gene was used to represent the nuclear DNA concentrations. Thermal cycles of PCR were as follows: 2 minutes of incubation at 50°C, followed by a first denaturation at 95°C for 10 minutes, and 40 cycles of incubation at 95°C for 15 seconds and 60°C for 1 minute. All measurements were performed in duplicates for each sample. The comparative threshold cycle (Ct) method was used to analyze data. The ΔCt values of each sample were obtained by subtracting the value of the endogenous control gene. The $2^{-\Delta\Delta Ct}$ was used to calculate the relative amounts, which were presented as the fold difference between two fetuses in MC twins.

Mitochondrial Staining with MitoTracker

MitoTracker (Invitrogen) is a fluorescent dye that labels mitochondria within live cells, and its accumulation is dependent on mitochondrial membrane potential. Mitochondria of the human AFMSCs were stained with MitoTracker dye at 37°C in 5% CO_2 for 30 minutes. Then, the cells were fixed with 4% paraformaldehyde in PBS for 10 minutes, and the nuclei were stained with 6-diamino-2-phenylindole (DAPI; Molecular Probes, Invitrogen). The slides were mounted with a mounting medium (Vector Laboratories) and examined with a laser scanning confocal microscope (Leica). Mitochondrial fluorescent intensities were analyzed with the Image J 1.45s program (Wayne Rasband, National Institutes of Health), as previously described (23).